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Studies on the Straub Diaphorase. II. Properties of an Antibody to the Straub Diaphorase*

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ABSTRACT: Rabbit antibody has been prepared against a single fraction of the Straub diaphorase obtained by chromatography on DEAE-cellulose. The antibody reacts in the Ouchterlony double-diffusion test to give a line of identity with all chromatographic forms of the enzyme. In the immune precipitate the flavin fluorescence is only slightly affected; markedly variable inhibition of the several reactions catalyzed by this enzyme is obtained.

Although a variable fraction of the flavin bound to the immune precipitate is not reducible by reduced diphosphopyridine nucleotide, the evidence suggests that the inhibition of enzymatic activity in the immune precipitate is associated principally with a modification of the reactivity of the bound flavin–adenine dinucleotide.

The Straub diaphorase, identical with lipoyl dehydrogenase (Massey, 1958, 1960b; Searls and Sanadi, 1959, 1961), has been separated into several electrophoretic components (Atkinson *et al.*, 1962) with variable enzymatic activities. A homologous liver enzyme has been separated into several fractions on DEAE-cellulose and DEAE-Sephadex by Lusty (1963), who also carried out starch-gel electrophoretic subfractionation of the column fractions. The first paper (Stein and Stein, 1965) in this series has reported the separation on DEAE-cellulose of the pig heart enzyme into three separate fractions which react identically in several enzymatic tests.

This study was undertaken to explore the immunochemical relationships between the three peaks; in the tests applied thus far, these appear to be identical. Some of the properties of the immune reaction and of the immune precipitate are described here.

Experimental

Enzyme Preparations. The procedures used to prepare and separate the Straub diaphorase into several chromatographic fractions were described in the first publication in this series. The DEAE-cellulose fractions resulting from the chromatography both of the free form of the enzyme and from the urea-resolved complex were used in this study.

Preparation of Immune Globulins. Two separate samples of peak I, with spectral ratios of 280/455 mμ of about 5.25, were used as antigens. Doses (10 ml) of 10–15 mg protein, emulsified in an equal volume of incomplete Freund's adjuvant, were injected in two separate sites, both gastrocnemius muscles, of two large New Zealand rabbits. The first dose was followed by two doses at intervals of 1 month. Samples of serum were obtained immediately before and a month following the third antigenic dose. The sera were fractionated between 0.3 and 0.5 saturated ammonium sulfate; the globulins were reprecipitated with ammonium sulfate and the fractionations were repeated. In the final preparation the globulin solutions represented approximately a 2-fold increase in concentration over

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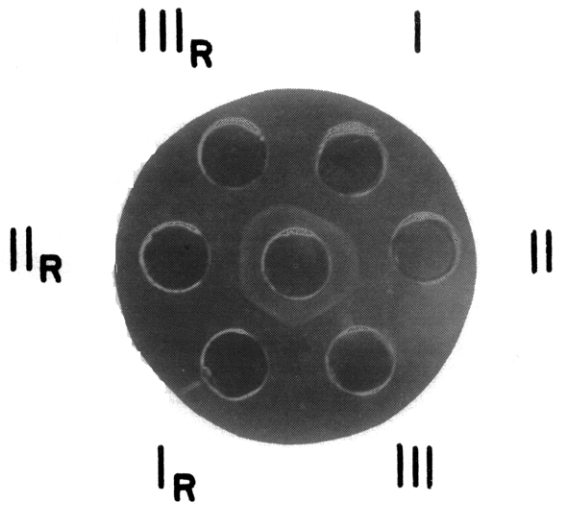

FIGURE 1: The reaction of DEAE-cellulose chromatographic fractions of the Straub diaphorase with rabbit immune globulin. Center well contains 0.05 ml precipitating globulin, the outside wells contain the enzyme fractions. The subscript "R" refers to the chromatographic fractions derived from the urea-resolved complexes.

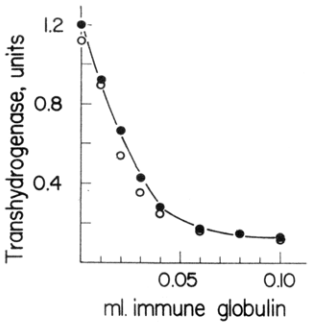

FIGURE 2: Titration of transhydrogenase activity with immune globulin. The enzyme (solid circles, 2 μl of peak I; open circles, 2 μl of peak III) was allowed to react with the indicated volume of immune globulin in 1-ml reaction mixtures, 0.1 M NaCl, 0.05 M Tris-chloride, *p*H 7.4, 10^{-4} M EDTA, for 10 minutes. Aliquots were assayed in the standard transhydrogenase reaction mixture; the activity is expressed in terms of the units recovered in the reaction mixtures. The line is drawn through the points derived from peak I.

the initial sera. The serum and derived globulin fraction obtained from one rabbit gave evidence of low concentration of precipitating antibodies; the globulin fraction obtained from the other rabbit yielded a strong precipitation reaction with an enzyme activity neutralization equivalence of 0.2–0.25 mg of enzyme protein per ml of globulin solution. Data obtained with this sample of globulin are reported exclusively in this paper.

Materials. DPNH[1] and DCIP were obtained from the Sigma Chemical Co.; TNDPN was prepared as described previously (Stein *et al.*, 1963); DL-lipoamide was obtained from Farmaco Cutolo-Calosi of Naples; coenzyme Q_0 was a gift from Dr. Britton Chance. Ionagar No. 2, a purified agar-agar product, was obtained from the Consolidated Laboratories, Inc., Chicago Heights, Ill.

Methods. Enzyme assays: The transhydrogenase reaction,

$$\text{DPNH} + \text{TNDPN}^+ \rightarrow \text{DPN}^+ + \text{TNDPNH}$$

was measured as described by Stein *et al.* (1959) but with 10^{-5} M EDTA instead of *o*-phenanthroline; the diaphorase reaction was as described by Stein and Kaplan (1958) with a concentration of DCIP sufficient to produce an absorbance of 1.8–2.0 at 600 mμ. Lipoyl dehydrogenase activity was measured under the conditions described by Massey *et al.* (1960). Coenzyme Q_0 reductase activity was measured by following the change in concentration of DPNH at 340 mμ (Stein and Stein, 1965). Estimation of the rates are based on the first 15–30 seconds of the reaction. All reactions were followed spectrophotometrically on the Model 2000 Gilford recording spectrophotometric system. All activities units are expressed in micromoles of substrate oxidized or reduced per minute.

Fluorescence spectra and rates of reduction of protein-FAD fluorescence were measured on an Aminco-Keirs spectrophosphorimeter provided with an *x–y* recorder.

Immunodiffusion tests were carried out as described by Ouchterlony (1962); 5 μg of enzyme was detected easily using 0.85% sodium chloride buffered with 0.05 M Tris-chloride, *p*H 7.0, supplemented with 1 mM EDTA in the diffusion medium.

Results

The reaction of anti–peak I globulin with the six chromatographic fractions of the pig heart Straub diaphorase is shown in Figure 1. In the double-diffusion test lines of identity for all fractions are formed about the antibody well, suggesting that all fractions are immunochemically equivalent. This result is typical of that obtained in several geometrical patterns combining the peaks in different orders. Additional evidence for the immunochemical identity of the chromatographic forms of the enzyme is obtained by titrating the transhydrogenase activity of the peaks with the precipitating globulin, as is shown in Figure 2. It is evident that the two antigens behave identically in the quantitative neutralization test; further, both peaks yield about the same end point in double-diffusion analysis (about 0.03–0.04 ml of immune globulin using the criterion of sharpest line of precipitation [Ouchterlony, 1962]).

[1] Abbreviations used in this report are: DPN, diphosphopyridine nucleotide; DPNH, reduced DPN; TNDPN, the thionicotinamide analog of DPN; DCIP, 2,6-dichlorophenolindophenol; FAD, flavin–adenine dinucleotide.

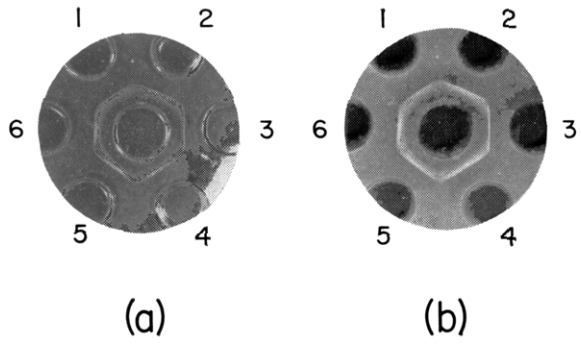

FIGURE 3: Fluorescence of the immune precipitate. Well contents were allowed to diffuse for 1 week. The plate was washed over a period of several days with changes of saline followed by changes in distilled water. The plate was photographed (a) under ordinary fluorescent lamp illumination and (b) under an ultraviolet lamp in a darkened room. Center well contains 0.05 ml precipitating globulin; side wells 2, 4, and 6 contain peak I; well 3 contains peak III; wells 5 and 1 contain unfractionated mixtures of the flavoproteins derived from the 0.45 saturated ammonium sulfate–soluble and urea-resolved 0.45 saturated ammonium sulfate–insoluble fractions, respectively.

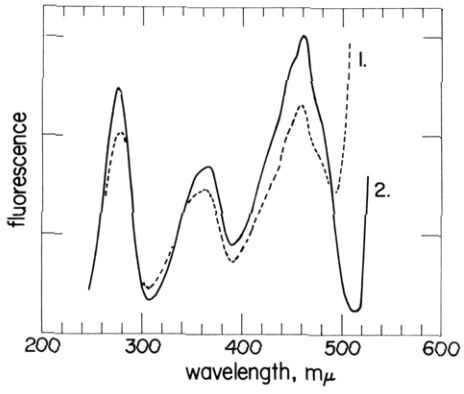

FIGURE 4: Excitation spectrum of the Straub diaphorase and its complex with antibody. Emission is measured at 550 mμ. (1) Immune precipitate; (2) peak III. Samples prepared in 0.85% NaCl, 0.1 M Tris-chloride, *p*H 7.7, and 10^{-4} M EDTA.

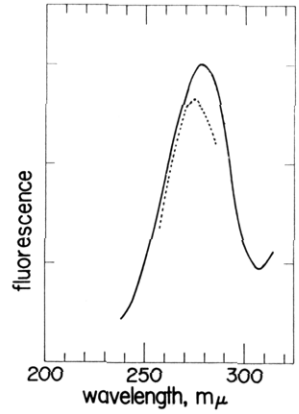

FIGURE 5: Excitation spectrum of the Straub diaphorase and its complex with antibody in the ultraviolet region. Conditions as in Figure 4. Concentrations were adjusted so as to give similar yields; each curve was traced four times. Dotted line, free enzyme; solid line, immune precipitate 2 (see Table I).

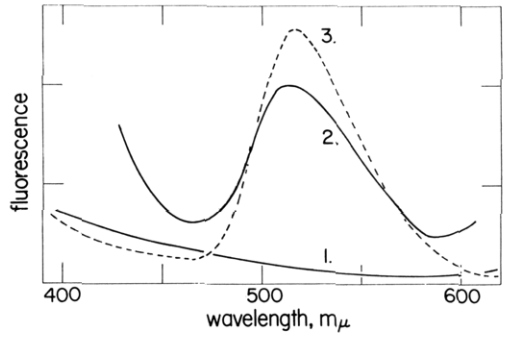

FIGURE 6: Emission spectrum of the Straub diaphorase and its complex with antibody. (1) Base line; (2) immune precipitate; (3) peak III. Conditions as in Figure 4.

The unusual fluorescence of this flavoprotein was first noted by Straub (1939) and studied by Palmer and co-workers (see Massey, 1963). It was of some interest, therefore, to determine whether the immune precipitate in agar is itself fluorescent: examination of the double-diffusion plates under an ultraviolet lamp in a darkened room reveals a fluorescent pattern identical to that due to the light scattering of the precipitate as viewed under cross-illumination. This effect is shown in Figure 3. It will be noted that only a single line of precipitation is obtained using an impure antigen, evidence for the specificity of the antibody. Determination of the specificity of the antibody in this system is thus aided by the fluorescence of the immune precipitate: increasing the concentration of peak I in the antigen well 20- to 50-fold produces an additional very faint and rather diffuse line of precipitation which is nonfluorescent. Since this reaction is obtained with peak III as well, its significance is somewhat obscure. Fluorescence of the immune precipitate suggests that the antibody interacts with the protein in a manner such as not to interfere with the flavin fluorescence. Measurement of the fluorescence of a sample of enzyme excited at 460 mμ before and after the addition of excess antibody shows no significant effect on light emission at 550 mμ (about 3% decrease). In studies on the old yellow enzyme, Kistner (1958, 1960) has shown that rabbit antiserum, inhibiting enzyme activity, was not directed against the flavin moiety of the enzyme.

TABLE I: Enzymatic Activities of the Immune Precipitate of the Straub Diaphorase.

Reaction	Unmodified Enzyme[a]	Immune Precipitates 1 Peaks I + III	Immune Precipitates 2 Peak III	Immune Precipitates 3 Peak III	Average Inhibitions of Precipitates 1 and 3 (%)
DPNH + lipoamide	1	1	1	1	98
DPNH + TNDPN	0.43	2.45	0.94	2.05	92
DPNH + DCIP	0.015	0.91	0.14	0.94	8
DPNH + CoQ_0	0.57	3.44	1.0	4.01	84
Fraction of transhydrogenase activity[b]	1	0.12		0.041	
Fraction of flavin reducible by DPNH[c]			0.76	0.52	

[a] The figures are averages of the values obtained for peaks I and III (Stein and Stein, 1965). [b] Value expected on the basis of sample fluorescence. [c] Fraction of $Na_2S_2O_4$-reducible fluorescence reduced by DPNH.

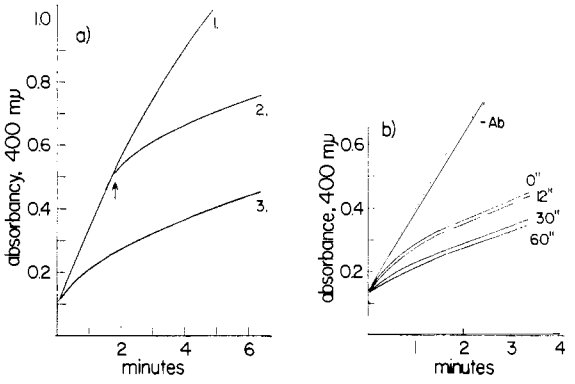

FIGURE 7: The effect of pyridine nucleotides on the inhibition by antibody of the transhydrogenase reaction catalyzed by peak I. (a) Reactions started with enzyme: (1) control reaction; (2) 0.03 ml antibody added at arrow; (3) 0.03 ml antibody in reaction mixture at zero time. (b) Reactions started with TNDPN: Enzyme and DPNH preincubated in cuvet with excess immune globulin for indicated number of seconds; the reaction is started by the addition of TNDPN. Control reaction is curve marked "—Ab." Rates were measured on a Cary spectrophotometer, Model 14.

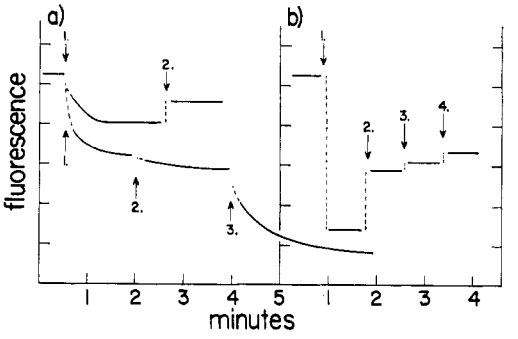

FIGURE 8: Reduction of flavin in the immune precipitate and free enzyme by DPNH. Cuvet temperature, 7–10°; reaction mixtures gassed with nitrogen 15 minutes before measurements were taken. Excitation and emission wavelengths, 470 and 520 mμ, respectively. (a) Immune precipitate. Additions: upper trace, (1) 20 μg DPNH, (2) 300 μg DPN; lower trace, (1) 200 μg DPNH, (2) 200 μg DPNH, (3) 3000 μg $Na_2S_2O_3$. (b) Peak I. Additions: (1) 20 μg DPNH, (2, 3, 4) each 200 μg DPN.

To study the fluorescence, as well as other properties of the product of the immune reaction, immune precipitates were prepared. The enzyme samples, in 0.85% sodium chloride, 0.01 M Tris-chloride, *p*H 7.4, and 1 mM EDTA, were treated for 3–4 hours at room temperature with an amount of immune globulin equivalent to the enzyme in the transhydrogenase activity neutralization test. After storage at 2–4° for several days, the precipitate was centrifuged, washed six times, and resuspended in the same cold buffer. Suspensions of moderate stability were thus formed, which permitted reproducible aliquoting and observation in the spectrofluorometer cuvet. The excitation and emission spectra of the unmodified protein and the immune precipitate are shown in Figures 4, 5, and 6. The spectra appear to be identical, except for a small shift of 5–7 mμ to longer wavelengths in the ultraviolet excitation band. The position of the excitation maximum of the immune precipitate does not change over a 4-fold range in concentration.

Given the electron acceptors operative with this enzyme, it was of interest to compare the profile of activi-

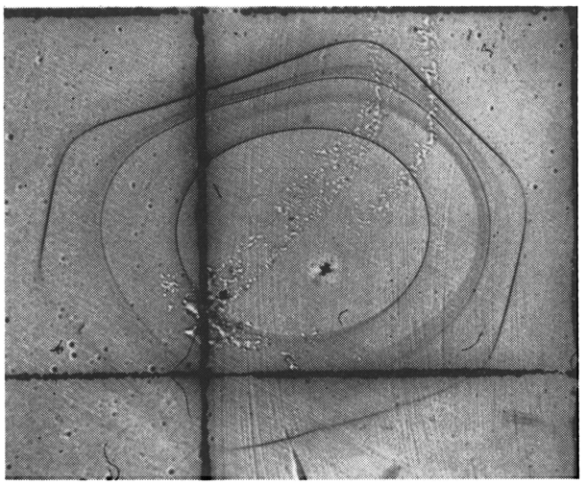

FIGURE 9: Double diffusion of peak I and anti–peak I globulin on cellulose acetate. The filter disks (Oxoid membrane filters, 5 cm) were soaked several hours in the diffusion medium described in the experimental section; 2 μl of antipeak I was deposited in the central position and 1 μl of peak I was deposited in each of the peripheral positions. The system was allowed to diffuse for 2 days in a moist box at room temperature. The disk was then extracted with saline and fixed in 3% trichloroacetic acid–0.2% Ponceau R. The line spacings are approximately 7 mm apart.

ties of the immune precipitate with that of the native enzyme. As is shown in Table I, the relatives rates of the various reactions change markedly in the immune precipitate; lipoyl dehydrogenase is almost completely inhibited, whereas the reduction of DCIP remains relatively unaffected. The effect on transhydrogenase and quinone reductase is intermediate. The residual transhydrogenase activity of the washed immune precipitate approximates that which is obtained in the neutralization of activity with antibody (Figure 2).

The spectral modification of the Straub diaphorase in reduction by DPNH has been demonstrated by Savage (1957) and by Massey *et al.* (1960). In addition, DPNH (a) generates a cadmium-sensitive site on the enzyme (Stein *et al.*, 1960) which binds cadmium in a ratio of 1 g-atom of cadmium per mole of flavin[2] and is identifiable with the formation of two newly titratable sulfhydryl groups (Searls *et al.*, 1961; Palmer and Massey, 1962); (b) renders the enzyme sensitive to denaturation by urea (Massey *et al.*, 1962), suggesting modification of protein conformation. Consequently, it was of interest to explore the interaction of antibody and enzyme reduced by DPNH. A preliminary experiment is reproduced in Figure 7, demonstrating the effect of the addition of a large excess of antibody to the transhydrogenase reaction mixture. A progressive inhibition of the rate of reaction is obtained, with a new limiting rate approached 2–3 minutes after the addition of antibody (Figure 7a). The time of addition of antibody does not appear to affect materially the course of inhibition. Development of inhibition appears to occur in much the same manner upon addition of antibody to the incomplete reaction mixture containing DPNH and enzyme or (not shown) TNDPN and enzyme (Figure 7b). It should be noted that no significant effect ($\leq 3\%$) on the rate of reaction is obtained upon the addition of globulin absorbed by an equivalent amount of peak III.

The occurrence in the immune precipitate of a fluorescent FAD group which participates in the enzymatic redox cycle permits evaluation of enzyme-antibody binding in terms of the reducibility of the enzyme by suitable electron donors. Figure 8 shows data obtained with immune precipitate 3 (Table I), following reduction of flavin by decrease in fluorescence.[3] In this case, DPNH yields about one-half the fluorescence decrease produced by sodium hydrosulfite. While the rate of reduction of the flavin of the uncomplexed enzyme is too rapid to be followed by this method (Massey *et al.*, 1960), the reduction of flavin bound to the immune precipitate is demonstrated to proceed at a rate slower by at least one to two orders of magnitude. Of considerable interest is the demonstration of the free reversibility of the reaction on the addition of DPN. These data, as well as others obtained at different concentrations of pyridine nucleotides, further suggest that the potential of the flavin may be modified in the combination of enzyme and antibody.

Discussion

The results of the double-diffusion and quantitative neutralization tests suggest that the chromatographic forms of the Straub diaphorase are immunochemically equivalent. This contrasts with the results obtained with other systems of multiple enzyme forms: lactic dehydrogenases (Pesce *et al.*, 1964; Fondy *et al.*, 1964); aldolases (Blostein and Rutter, 1963); and glutamic dehydrogenases (Talal and Tomkins, 1964a,b). It is possible that more sensitive immunochemical analyses might reveal differences between the various forms which underlie their different chromatographic mobility. Further separation of the DEAE fractions into multiple electrophoretic components has been achieved (Stein and Stein, 1965), and thus the immunizing antigen is in fact polydisperse. An examination of unbalanced systems in the double-diffusion reaction on agar gel has demonstrated the ready formation of blurred zones and multiple lines of precipitation which fuse into a single sharp line as equivalence is approached. A preliminary test of double diffusion on cellulose acetate disks using large antigen excess (Figure 9) has shown several lines of precipitation which, in number and size, recall the pattern of bands obtained in acrylamide-gel electrophoresis

[2] A. M. Stein, unpublished data.

[3] Not evident in Figure 8 is an approximate doubling of the fluorescence yield with a decrease in temperature of the sample from ambient (22°) to that of the thermostated cuvet. This effect has been noted by Massey (1963).

(Stein and Stein, 1965). It is possible that these effects may reflect inhomogeneity of the antibody complementary to the electrophoretic inhomogeneity of the fraction employed, and not merely a case of Liesegang ring formation (Ouchterlony, 1962).

The properties of the immune reactions of this system are of interest since the flavin fluorescence may be monitored with facility to follow the state of enzyme reduction in systems comprising enzyme, antibody, reducing agent, and electron acceptor. The data reported above suggest that, in the combination with enzyme, the antibody reacts slightly if at all with the catalytic site(s) of the protein (Cinander, 1963). The slight shift in excitation maximum of the immune precipitate may be attributed to a change in conformation leading to increased energy transfer from enzyme protein to FAD, although a direct effect of antibody protein is certainly not excluded. Pyridine nucleotides offer little protection to the enzyme against inactivation by antibody, although it will be seen that the data of Figure 7 do not exclude completely a small protective effect of substrate.

The variation in profile of enzymatic activities in the immune precipitate is reminiscent of the variation of the reactions catalyzed by immune precipitates of chicken lactic dehydrogenases (Kaplan and White, 1963). Of interest is the observation that the rate of reduction of FAD in the immune precipitate is of the same order of magnitude as the reductions of lipoamide, TNDPN, and coenzyme Q_0. The anomalous insensitivity of the diaphorase reaction to reaction of the enzyme with antibody is of interest, since it provides a further criterion for discrimination between this activity and lipoyl dehydrogenase and transhydrogenase activities, such as is obtained in treatment of the enzyme with copper (Massey, 1960a). The properties of the immune precipitate. suggest that the FAD moiety of enzyme is not involved in the catalysis of the reduction of DCIP by DPNH.

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